

Experimental Transmission of Karshi and Langat (Tick-Borne Encephalitis Virus Complex) Viruses by *Ornithodoros* Ticks (Acari: Argasidae)

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J. Med. Entomol. 41(5): 973-977 (2004)

ABSTRACT Selected species of mosquitoes and *Ornithodoros* ticks were evaluated for their potential to transmit Karshi and Langat (tick-borne encephalitis virus complex) viruses in the laboratory. Although there was no evidence of replication of Karshi virus in either of the two mosquito species tested [*Ochlerotatus taeniorhynchus* (Wiedemann) or *Culex pipiens* (L.)], Karshi virus replicated in and was transmitted by all three species of *Ornithodoros* ticks tested (*Ornithodoros parkeri* Cooley, *Ornithodoros sonrai* Sautet & Witkowski, and *Ornithodoros tartakovskyi* Olenov). When inoculated with Karshi virus, 90% of *Ornithodoros* ticks (44/49) transmitted this virus by bite to suckling mice, and transmission continued to occur for at least 1 yr, the longest extrinsic incubation tested. After feeding on a suckling mouse with a viremia of $\approx 10^5$ suckling mouse subcutaneous lethal dose₅₀ units of Karshi virus per milliliter of blood, all three species of *Ornithodoros* tested became infected with and transmitted Karshi virus both trans-stadially and horizontally by bite to suckling mice. In addition, female *O. tartakovskyi* transmitted Karshi virus vertically to their progeny. In a continuation of a previous study, *O. sonrai*, orally exposed to Langat virus, were able to transmit this virus after >3 yr, the longest interval tested. Therefore, *Ornithodoros* spp. should be considered as potential vectors and as possible long-term maintenance hosts for Karshi virus and other members of the tick-borne encephalitis virus complex.

KEY WORDS vector competency, tick-borne encephalitis virus, *Ornithodoros*, mosquitoes, Karshi virus

KARSHI AND LANGAT VIRUSES are members of the tick-borne encephalitis (TBE) virus complex (genus *Flavivirus*, family *Flaviviridae*) (Calisher 1988). Although neither Karshi nor Langat viruses are considered to be highly pathogenic, infections in healthy persons may cause a febrile illness (Gritsun et al. 2003). Severe disease, including encephalitis, has been reported in a few individuals, and large outbreaks of febrile illness associated with infection with Karshi virus have been observed in Uzbekistan (S.K., unpublished data).

The natural transmission cycle of TBE complex viruses involves ixodid ticks and rodents (Gresikova and Calisher 1989). Karshi virus originally was isolated from *Ornithodoros tholozani* (Laboulbene and Megnin) (reported as *Ornithodoros papillipes* Birula) ticks in Uzbekistan, and Langat virus has been isolated from a variety of ixodid ticks, including *Ixodes granulatus*

Supino collected on forest rats [*Rattus mulleri validus* (Miller) and *Rattus sabanus vociferans* (Miller)] in Malaysia, *Hemaphysalis papuana* Thorell in Thailand, and *Ixodes persulcatus* Schulze in central Siberia (Smith 1956, Bancroft et al. 1976, Karabatsos 1985). Both ixodid, *Dermacentor marginatus* (Sulzer), *Ixodes ricinus* (L.) and *Hemaphysalis spinigera* Neumann (Varma and Smith 1962, Karabatsos 1985), and argasid, *Ornithodoros sonrai* Sautet & Witkowski (Turell and Durden 1994), ticks transmit Langat virus in the laboratory. In addition, Karshi virus was reported to be transmitted by *Aedes aegypti* (L.) mosquitoes in a laboratory experiment (Khutoretskaya et al. 1985). Rodents may serve as reservoir hosts for Karshi and Langat virus in nature (Smith 1956, Karabatsos 1985). Although experimental studies on members of the TBE virus complex have focused on ixodid ticks, several members of the TBE virus complex, in addition to Karshi virus, have been isolated from *Ornithodoros* ticks (Rajagopalan et al. 1969, Dhanda and Rajagopalan 1971, Chastel et al. 1985, St George et al. 1977). The association of several TBE-complex viruses with argasid ticks in nature, the demonstration that these ticks can transmit Langat virus (Turell and Durden

The views of the authors do not necessarily reflect the position of the Department of Defense or the Department of the Army.

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Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01 MAY 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Experimental transmission of Karshi and Langet (tick-borne encephalitis virus complex) viruses by Ornithodoros ticks (Acari: Argasidae), Journal of Medical Entomology 41:973 - 977				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Ulrich, RL				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD				8. PERFORMING ORGANIZATION REPORT NUMBER RPP-04-233	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Selected species of mosquitoes and Ornithodoros ticks were evaluated for their potential to transmit Karshi and Langat (tick-borne encephalitis virus complex) viruses in the laboratory. Although there was no evidence of replication of Karshi virus in any of the mosquito species tested ([Aedes albopictus (Skuse), Ochlerotatus taeniorhynchus (Wiedemann), or Culex pipiens (L.)], Karshi virus replicated in and was transmitted by all three species of Ornithodoros ticks tested (Ornithodoros parkeri Cooley, Ornithodoros sonrai Sautet & Witkowski, and Ornithodoros tartakovskyi Oleney). When inoculated with Karshi virus, 90% of Ornithodoros ticks (44/49) transmitted this virus by bite to suckling mice, and transmission continued to occur for at least 1 yr, the longest extrinsic incubation tested. After feeding on a suckling mouse with a viremia of ~105 suckling mouse subcutaneous lethal dose50 units of Karshi virus/ml of blood, all three species of Ornithodoros tested became infected with and transmitted Karshi virus both transstadially and horizontally by bite to suckling mice. In addition, female O. tartakovskyi transmitted Karshi virus vertically to their progeny. In a continuation of a previous study, O. sonrai, orally exposed to Langat virus, were able to transmit this virus after more than 3 years, the longest interval tested. Therefore, Ornithodoros spp. should be considered as potential vectors and as possible long-term maintenance hosts for Karshi virus and other members of the tick-borne encephalitis virus complex.					
15. SUBJECT TERMS tick-borne encephalitis virus, TBE, Karshi, laboratory transmission, vector, Ornithodoros ticks					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 5	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

Table 1. Transmission of Karshi virus by *Ornithodoros* ticks by time after feeding on mice with a mean viremia of $10^{5.3}$ SMLD₅₀ per milliliter

	Temp. (°C)	Days after viremic blood meal						Total
		4-30	31-60	61-90	91-120	121-150	151-180	
<i>O. parkeri</i>	20	—	—	—	—	—	78 (9) ^a	78 (9)
<i>O. sonrai</i>	20	—	—	0 (10)	—	100 (1) ^b	50 (14)	32 (25)
<i>O. sonrai</i>	26	100 (2) ^c	67 (3)	—	50 (2)	—	—	71 (7)
<i>O. tartakovskyi</i>	20	—	—	0 (2)	0 (5)	—	80 (5)	33 (12)

—, not tested.

^a Percentage transmitting virus by bite (no. of ticks feeding). Each feeding consisted of one tick on one mouse.

^b In addition to one of one individual ticks transmitting virus, one of two pools of four ticks each transmitted virus by bite on day 149 (data not included in totals).

^c In addition to two of two individual ticks transmitting virus, two of two pools of three ticks each transmitted virus by bite on day 29 (data not included in totals).

1994), and their association with rodents indicate that *Ornithodoros* spp. ticks may play a role in the natural maintenance of TBE-complex viruses. Therefore, we examined the potential for *O. sonrai*, *Ornithodoros parkeri* Cooley, and *Ornithodoros tartakovskyi* Olenov ticks as well as two species of mosquitoes to transmit Karshi virus. In addition, we report on some previously unpublished data on the potential for *O. sonrai* to transmit Langat virus after extended incubation periods.

Materials and Methods

Ticks. A laboratory colony of *O. sonrai* was established from wild-caught specimens excavated from mammal burrows in the Bandia Forest, Senegal, in 1989 (Turell and Durden 1994). No virus was detected upon examination of parental ticks from this colony. The *O. parkeri* were from a colony at Georgia Southern University derived from specimens captured in Spicer City, CA, in 1965. The National Institute of Allergy and Infectious Diseases provided a laboratory colony of *O. tartakovskyi* ticks, and all three colonies were maintained as described by Durden et al. (1993).

Mosquitoes. Specimens from established (>40 generations each) laboratory colonies of *Ochlerotatus taeniorhynchus* (Wiedemann) (Medical and Veterinary Entomology Research Laboratory strain) and *Culex pipiens* (L.) (New York 1999 strain) were used. Mosquitoes were reared in an insectary maintained at 26°C, and larvae were fed ground fish food.

Virus and Virus Assays. The U2-2247 strain of Karshi virus had been passaged once in Vero cells and once in suckling mice before use in these experiments. The TP-21 strain of Langat virus, obtained from the Yale Arbovirus Research Unit, was originally isolated from *I. granulatus* collected in the Ulu Langat Forest reserve, Malaysia, in 1956 (Smith 1956). It had been passaged eight times in suckling mice and three times in Vero cells before use in these experiments.

Serial dilutions of blood, brain, and tick samples were tested for virus by plaque assay on confluent monolayers of 2- to 3-d-old primary chicken embryo cells or by subcutaneous inoculation into 2- to 4-d-old suckling mice. The identity of the original virus, and virus recovered from ticks and mice, was confirmed by a Karshi-specific TaqMan polymerase chain reaction

(PCR) assay and by direct sequencing of the PCR products.

The primers (850f: 5-TGCCGATGGAGTGTCA-GAGA, 909r: 5-CAGCCACATCTGCCATAAG) and probe (871t: 5-ACAGCTTGTCTGTCAAAG) were designed using known sequence of the Karshi NS5 gene available in GenBank. A single-tube RT-PCR assay was used containing Invitrogen Superscript One-Step reagents supplemented with 1 μ M each primer, 0.25 μ M probe, 0.625 mM MgCl₂, 10 U of RNaseOUT (Invitrogen, Carlsbad, CA), and 800 ng of bovine serum albumin. Reactions were performed on an Idaho Technologies Ruggedized Advanced Pathogen Identification Device by using the following thermocycling profile: 15 min at 50°C, 5 min at 95°C, 45 cycles of 1) 5 s at 95°C and 2) 1 min at 60°C.

Experimental Design. One-day-old suckling mice (BALB/c strain) were inoculated intraperitoneally with $10^{6.3}$ suckling mouse lethal dose₅₀ (SMLD₅₀) units of Karshi virus. Two or 3 d after inoculation, a Karshi virus-inoculated mouse was placed in a cage containing ≈ 50 *O. sonrai*, *O. parkeri*, or *O. tartakovskyi* ticks at various stages of development (larvae through adult, but predominately early nymphs). After the ticks had been allowed to attach to the mouse for ≈ 5 min, the mouse was removed and a second virus-inoculated mouse was added to the cage. This was repeated for up to three mice for each species of tick used in this study. The ticks were allowed to feed on the virus-inoculated mouse for ≈ 2 h. Those that had attached and did not feed were removed and discarded. Each mouse was then killed with CO₂ and 0.1 ml of blood was collected by cardiac puncture. Blood was mixed 1:10 in diluent (Medium 199 with Earle's salts containing 10% heat-inactivated fetal bovine serum and 5 μ g of amphotericin B, 50 μ g of gentamicin, 100 U of penicillin, and 100 μ g of streptomycin per ml and 0.075% NaHCO₃) and frozen at -70°C until tested to determine the viremia at the time of tick feeding. The engorged ticks were placed in a cage maintained at room temperature ($\approx 20^\circ\text{C}$) or in an incubator maintained at 26°C until being tested for either infection or for the ability to transmit virus by bite. Ticks that had not attached to a virus-inoculated mouse were inoculated intracoelomically with 10^4 SMLD₅₀ ($10^{7.5}$ SMLD₅₀/ml) of the same virus

Table 2. Transmission of virus by *Ornithodoros* ticks by time after intracoelemic inoculation of 10^4 SMLD₅₀ of Karshi virus

Species	Temp (°C)	Days after inoculation							Total
		8	31-60	61-90	91-120	121-150	151-180	>181	
<i>O. parkeri</i>	26°	0 (3) ^a	—	—	—	100 (4)	100 (1)	—	63 (8)
<i>O. sonrai</i>	20°	—	83 (6)	100 (1)	—	100 (6)	100 (1)	—	95 (19)
<i>O. tartakovskyi</i>	20°	—	83 (6)	—	100 (3)	100 (1)	—	100 (12)	95 (22)

—, not tested.

^a Percentage transmitting virus by bite (no. of ticks feeding).

strain that had been used to infect the mice. These inoculated ticks were treated in the same manner as the engorged ticks. Similarly, uninfected *Oc. taeniorhynchus* and *Cx. pipiens* were inoculated intrathoracically (Rosen and Gubler 1974) with 10^4 SMLD₅₀ of Karshi virus and held in an incubator maintained at 26°C until tested for infectious virus.

To determine transmission rates, virus-exposed ticks or mosquitoes were allowed to feed for up to 2 h on naive suckling mice either individually [one tick or mosquito per mouse] or in some cases in small pools of up to four orally exposed ticks per pool. These suckling mice were marked by subcutaneous inoculation of India ink, returned to their dam, and then monitored daily over the next 21 d for signs of viral infection. Each litter contained one or two suckling mice that were either unexposed to ticks or were fed upon by uninfected colony ticks to serve as negative controls. Moribund mice were killed with CO₂, and brain samples were obtained and then triturated (1:10) in diluent and frozen at -70°C until tested for virus. Mosquitoes were killed by freezing at -20°C for 5 min, triturated in 1 ml of diluent, and then frozen until tested for the presence of virus. In most of the tick transmission trials, ticks were caged individually in plastic vials (12 ml, about half filled with washed sea sand) after feeding on the mice. Many of these same ticks were tested a second time for their ability to transmit virus by bite or to transmit virus vertically to their progeny.

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Table 3. Transmission rates for individual *O. sonrai* by time after feeding on mice with a mean viremia of $10^{7.2}$ plaque-forming units per milliliter and held at 21°C

	Months after viremic blood meal							Total
	<6	6-12	12-18	18-24	24-30	30-36	>36	
No. tested	33	29	20	6	6	2	3	99
Transmission rate	100	100	95	100	100	100	67	98

Only ticks that had previously transmitted virus by bite are included in this table.

Results

Oral Exposure Experiments. Viremias in mice at the time of the tick feedings were $\approx 10^{5.3}$ SMLD₅₀/ml. Initial experiments were carried out at an ambient temperature of $\approx 20^\circ\text{C}$. Although none of the ticks maintained at this temperature transmitted virus <149 d after the infectious blood meal, *O. sonrai* maintained at 26°C transmitted Karshi virus 29 d after the infectious blood meal (Table 1). When tested ≥ 149 d after the initial blood meal, at least half of orally exposed ticks for each species transmitted virus by bite (Table 1).

Inoculation Experiments. For both *O. sonrai* and *O. tartakovskyi*, five of six ticks transmitted virus by bite when tested 43 d after inoculation with Karshi virus. None of three *O. parkeri* transmitted virus by bite when tested 8 d after inoculation. However, all 34 ticks (five *O. parkeri*, 13 *O. sonrai*, and 16 *O. tartakovskyi*) tested at ≥ 64 d after inoculation transmitted Karshi virus by bite. Overall, 95% of both *O. sonrai* ($n = 19$) and *O. tartakovskyi* ($n = 22$) and 63% of *O. parkeri* ($n = 8$) transmitted Karshi virus by bite (Table 2). In contrast, we did not detect evidence of Karshi virus in any of the *Oc. taeniorhynchus* ($n = 11$) or *Cx. pipiens* ($n = 5$) when tested 8-43 d after inoculation with 10^4 SMLD₅₀ ($10^{7.5}$ SMLD₅₀/ml) of Karshi virus.

Vertical Transmission. Progeny were obtained from an adult female *O. tartakovskyi* that had fed on a suckling mouse 43 d after the tick had been inoculated with Karshi virus. The progeny were divided into three groups of ≈ 20 larvae each, and each of these groups was allowed to feed on a suckling mouse (≈ 20 ticks per mouse). Immediately after feeding, the engorged larvae from each group were placed in a plastic cage similar to the procedure described above, and the suckling mice were monitored for signs of virus infection. All three mice died, whereas the control mice showed no signs of virus infection. Approximately 45 d after this transmission attempt, 10 ticks from each of two of these three cages were then allowed to feed on a second set of suckling mice. In both cases, two of 10 ticks transmitted virus by bite, indicating trans-stadial infection, and a filial infection rate of 20%.

Langat. In a continuation of a study of *O. sonrai* orally exposed to the TP-21 strain of Langat virus by methods similar to those used in the current study (Turell and Durden 1994), 35 ticks that transmitted virus during their first transmission attempt were re-fed on naive mice for a total of 99 additional feedings.

These ticks transmitted virus during 97 (98%) of these refeeding attempts, including ticks that transmitted virus >3 yr after the initial infectious blood meal (Table 3).

Discussion

This is the first report of soft ticks becoming infected with and transmitting Karshi virus. Although only ixodid ticks have been implicated in the natural transmission cycle for TBE complex viruses (Gresikova and Calisher 1989), it is possible that soft ticks also are involved in the maintenance of these viruses in nature.

In our study, all three *Ornithodoros* spp. (*O. parkeri*, *O. sonrai*, and *O. tartakovskyi*) became infected with Karshi virus after feeding on viremic suckling mice and successfully transmitted this virus by bite. Because the natural distributions of these ticks cover western North America (*O. parkeri*) (Guglielmone et al. 2003) and sub-Saharan Africa (*O. sonrai*) (Colas-Belcour and Rageau 1962, Brès et al. 1967), as well as Karshi virus-endemic areas in southern states of the former Soviet Union, such as Uzbekistan (*O. tartakovskyi*) (Anastos 1957), the ability of *Ornithodoros* ticks to transmit Karshi virus seems to be a general characteristic of this genus. There was evidence for both transstadial and vertical transmission of Karshi virus by soft ticks and that these ticks maintained their ability to transmit virus for at least 1 yr (the longest interval tested). In a continuation of an earlier study with *O. sonrai*, individual ticks were able to transmit Langat virus >3 yr after their initial infectious blood meal. Because some soft ticks can survive for >10 yr (Oliver 1989), may take numerous blood meals, and are closely associated with rodents, *Ornithodoros* ticks could serve as long-term reservoirs for TBE-complex viruses. This is enhanced by the fact that *Ornithodoros* ticks can survive for 4 yr in sand without food or water (Anastos 1957), which would allow these ticks to survive after being infected by feeding on one viremic rodent until a second, uninfected rodent, enters that burrow years later. This would allow known TBE virus vectors (e.g., *I. persulcatus* and *I. ricinus*) to acquire infection by feeding on rodents infected by these *Ornithodoros* spp. ticks. Our failure to detect evidence of Karshi virus in any of the inoculated mosquitoes is additional evidence that this virus is maintained in nature by ticks.

Therefore, the susceptibility of *O. parkeri*, *O. sonrai*, and *O. tartakovskyi* to infection; their ability to transmit virus for extended periods; and the isolation of several members of the TBE-complex from *Ornithodoros* ticks (e.g., Karshi, Meaban, Kyasanur Forest, and Saumarez Reef viruses) (Rajagopalan et al. 1969, Dhanda and Rajagopalan 1971, St George et al. 1977, Chastel et al. 1985, Karabatsos 1985) indicate that *Ornithodoros* species should be studied further as potential reservoir hosts for members of the TBE-complex.

Acknowledgments

We thank M. L. Wilson (University of Michigan) for providing the *O. sonrai* for colony establishment; The National Institute of Allergy and Infectious Diseases for providing a colony of *O. tartakovskyi*; J. H. Oliver, Jr. and L. A. Durden (Georgia Southern University) for providing a colony of *O. parkeri*; D. L. Kline (Medical and Veterinary Entomology Research Laboratory) for providing the MAVERL strain of *Oc. taeniorhynchus*, and Kathy Kenyon for expert editorial assistance. This research was funded in part by the Biological Weapons Proliferation Prevention Program, Defense Threat Reduction Agency, Ft. Belvoir, VA. C.N.M. was supported by a research fellowship awarded by the National Research Council, Washington, DC.

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Received 27 February 2004; accepted 17 May 2004.